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THE EFFECT OF GENOTYPE ON ANDROGENESIS INDUCTION OF *AESCULUS HIPPOCASTANUM* L.

Dušica ĆALIĆ-DRAGOSAVAC[1], Snežana ZDRAVKOVIĆ-KORAĆ[1], Ljiljana RADOJEVIĆ[1]

Abstract: *Influence of different genotype, age of trees and environmental temperature on induction of androgenesis and apperance albino horse chestnut embryos were studied. Efficiency of in vitro androgenesis via anther and microspore culture were investigated using the same closed flower bud material. Androgenic response of different genotypes was measured and compared. Anther induction rates were from 5 % to 37.6 % depending of genotype. Under optimal conditions, the number of embryos per isolated anther varied between 0.5 to 5.0 embryos in anther culture, while in microspore culture varied between 3.0 to 27 embryos, depending of genotype. A microspore culture was 5-6 times efficiency than anther culture for same genotype.*

Age of the trees had no influence on androgenesis induction. Temperature of about 4-5 ^{0}C was optimal for androgenic embryo induction.

Flow cytometric analysis of embryos and regenerated plants showed that the most of the androgenic embryos were haploid, corresponding to their microspore origin, while a half of these were diploid, after 6 months in culture. However, diploid, tetraploid and octaploid embryos were observed after 3 years in subculturing.

Key words: age of tree, androgenic embryos, genotype, horse chestnut, ploidy stability

1. INTRODUCTION

Horse chestnut (*Aesculus hippocastanum* L., *Hippocastanaceae*) represent a relict species of the tertiary flora and endemit of Balkan peninsula. The development of anthers with uninuclear pollen grains leading to androgenesis and haploid plants orginating from one genotype has been described for *Aesculus hippocastanum* (Ćalić et al. 2003). Anther culture has been used in recent years as a tool for producing haploid plants in a varyety of higher plants, but the low frequencies of microspore-derived plants restrict the use of the technique in plant breeding. There are several factors affecting androgenesis in many species, such as genotypes, growth of donor plants, pretreatments of anthers, composition of medium and culture conditions (Assani et al. 2003; Hofer 2004). Androgenic response is genetically controlled and is affected by environmental factors. Genotype is the most crucial factor for androgenic response *in vitro* androgenesis in apple (Höfer 2004; Höfer *et al*. 2008). Also the developmental stage of microspores within anthers is an important factor for success in anther cultures (Perera et al. 2008; 2009). The anthers containing microspores at the uninuclear stage and the first pollen mitosis are determined to be optimal for the induction androgenesis for many woody plant species (Marinković and Radojević 1992; Assani et al. 2003; Germana 2006; Hofer 2004; Pintos et al. 2007). Radojević (1978, 1991) determined that horse chestnut anthers taken from buds at the size beetwen 3 and 7 mm containing microspores at the uninuclear stage and gave good results. Besides green embryos, albino embryos were also obtained, but their development in culture was slow. Although the occurrence of albino plants is a general phenomenon, extensive and systematic studies are presently lacking on this subject. The problem is particularly significant for plant breeding.

Stress treatments play a major role in androgenesis. The influence of temperature and nutrition in different crop species is well documented (Shariatpanahi et al. 2006).

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Most trees are characterized by a long reproductive cycle with several years of a juvenile phase, a tendency to allogamy and a large tree size. They are generally highly heterozygous, outbreeding species, which are asexually propagated (Zdravković-Korać, 2008). For this reasons, their genetic improvement by conventional methods is time/consuming and limited by space for field experiments. The production of haploids offers new possibilities for genetic studies and breeding (Hofer 2005). Biotechnological methods can improve the efficiency and increase the speedy of breeding. Anther culture is a widely used method to generate genetic variability. Production of haploid plants from anther cultures is specially useful for regeneration and breeding of forest trees, since the long regeneration time and strong inbreeding depression of these species makes the traditional breeding methods impractical. No extensive study has been done to characterise the process of determining the cellular origin of those late embryos, in long term-cultures, as well as ascertaining in which proportion the haploid condition occurs and genetic variability is present. Only recently has the been some preliminary data reported in *Q. suber* microspore-derived embryos (Bueno et al. 2000). It is well known that the use of *in vitro* techniques to induce androgenesis has significantly facilitated the production of doubled haploids in plant breeding programs, leading to the early release of homozygous lines. Several methods (e.g., anther and microspore culture) have been developed for the *in vitro* production of doubled haploids and these have been used in breeding programs of many plant species. Since the introduction of anther culture for the production haploid plants, the production of haploid plants has increased considerably (Bueno et al. 2003; Ćalić et al. 2003; Höfer 2004).

Flow cytometric analysis was used to verify the ploidy stability of the horse chestnut androgenesis process.

2. MATERIALS AND METHODS

Plants materials

Inflorescences were harvested from ten different genotypes (an approximately 20 and 100-years-old) of *Aesculus hippocastanum* L. trees growing in the Botanical garden ″Jevremovac″ of Belgrade University. Anthers were excised from closed flower buds (size 5 mm). In fact, a high correlation has been shown between flower lenght and phase of pollen development. In buds of 5 mm microspores were at the uninucleate stage of development. Determining the correct developmental stage of the microspores, were performed with 0.1 mg l^{-1} 4',6-diamidino-2-phenylindole (DAPI). Dimorphism determined with aceto-carmine while microspore viability with fluorescein diacetate (FDA).

Anther and microspore culture

The selected closed buds with uninucleate microspores were surface sterilized with 95 % ethanol (3 min) and 70 % ethanol (5 min) and three rinses in sterilized water. Basal medium (BM) contained MS mineral salts (Murashige and Skoog 1962), 2 % sucrose, 0.7 % agar, 100 mg l^{-1} myo-inositol, 200 mg l^{-1} casein-hydrolisate, 2 mg l^{-1} vitamin B_1, 10 mg l^{-1} pantothenic acid, 5 mg l^{-1} nicotinic acid and 2 mg l^{-1} adenine sulphate. Uninucleate microspores cultured in MS liquid medium (MSL) while anther culture establish on same solid medium (MSS) with 0.7 % agar. Liquid and solid MS media contained BM and 2,4-dichlorphenoxyacetic acid (2,4-D) and kinetin (Kin) about 1 mg l^{-1}. A fifthy dissected anthers with uninucleate microspores per Erlenmeyer flask with filter (100 μm) and 50 ml MSL medium for androgenesis induction. The microspore suspension was refreshed every 4 weeks with MSL medium. After 8 weeks, the suspension was plated by Bergmann technique (1959) on a solid MSS_1 medium reduced concentration of 2,4-D 0.01 mg l^{-1} and Kin 1 mg l^{-1}. Anthers were cultured on petri dish containing about 30 ml MSS induction medium. Development and multiplication of androgenic embryos orginating from

anther and microspore culture proceeded on MSS_1 medium. All media were sterilized by autoclaving at 0.9 x 10^5 Pa and 114 ^{0}C for 25 min. Suspension cultures were grown on a horizontal shaker (85 rpm) at temperature of 23 ± 1 ^{0}C for one month in the dark. All other cultures were grown at the same temperature with irradiance of 33-45 μmol $m^{-2}s^{-1}$ produced by cool white fluorescent tubes. Androgenic embryos were growth on MS hormone-free medium a 16/8 h light/dark and 8/16 h light/dark photoperiod.

Determination of ploidy level

Nuclear suspension from androgenic embryos in cotyledonary stage of development was prepared. Young leaf material of horse chestnut was used as control. Plant material was macerated with a sharp razor blade in a ice-cold neutral buffer, and placed in plastic Petri dishes. Neutral DNA buffer (pH 7) with 15 mM Hepes, 1 mM EDTA, 80 mM KCl, 20 mM NaCl, 0,5 mM spermine, 300 mM sucrose, 0.2 % Triton X-100, 15 mM DTE (Dithiothreitol) and 2 mg l^{-1} DAPI was used. After maceration, the buffered mixture (ca. 2 ml), was passed through a nylon filter of 40 μm mesh size, stained with DAPI, and analysed in a flow cytometer. Fluorescence levels were determined by a photomultiplier and coverted in voltage pulses that were processed with PC. Ploidy level of androgenic embryos was evaluated by flow citometry, using a PAS II cytometer (Partec GmbH), equipped with a high pressure mercury lamp (OSRAM HBO 100 W/2) and using the excitation filters UG-1, BG-31, KG-1 and TK-420 and emission filters TK560 and GG435.

Statistics and repetition

Influence of ten horse chestnut genotypes on androgenic embryo induction was investigated during one year. The sample size was about 201-210 embryos for each of ten genotypes. The number of obtained embryos per one anther and percentage of responding anthers were used as indicators of the efficiency of androgenesis. The results were assessed using the variation analysis. The means were compared by the SNK (Student Newman-Keuls test; significance level α= 0.05).

3. RESULTS

Genotypes JT_1 - JT_5 were 20 years old, while genotypes OT_1 - OT_5 were 100 years old. Anthers originating from young YT_1 and YT_3 genotypes had higher embryogenesis potential (3.0 and 2.7 embryos/anther) than those originating from genotypes YT_2, YT_4 and YT_5 (1.0; 0.9 and 1.6 embryos/anther, respectively), Table 1. However, anthers originated from old genotype OT_1 produced the largest number (5.0 embryos/anther), while anthers isolated from genotypes OT_3 and OT_5 produced the less number of androgenic embryos (1.0 and 0.5 embryos/anther; respectively), Table 1. All horse chestnut genotypes were having a high, but different androgenic responses. Results shown in Table 1, confirmed that microspore culture was 5-6 times efficient method for androgenesis induction than anther culture for all investigated genotypes. The number of embryos per isolated anther varied between 0.5 to 5.0 embryos in anther culture, while in microspore culture varied between 3.0 to 27 embryos, depending of genotype (Table 1). The frequency of albino androgenic embryos in hormone-free medium was also monitored. Similarly, the number of albino embryos produced per 210 anthers ranged between 4–14 % (LD) and 15–25 % (SD) in anther culture, while present of albino embryos in microspore culture was between 1-11 % (LD) and 11-24 % (SD), depending of genotype (Table 2). It was also observed that albino androgenic embryos formation is significantly increased in the short, in comparison to a long day.

Cytogenetic analysis of androgenic embryos originating from anther and microspore culture was done after a first generation of regenerants and after 3 years of subculturing. All androgenic embryos the first generation from microspore culture were haploid. Immediately after germination, 50 % of the regenerants originating from anther culture were haploid, and the other half diploid. After 3 years of subculturing, there were no haploid regenerant from anther culture, while 8.5 % were diploid, 81 % tetraploid and 10.5 % octaploid. Unlike those from anther culture, all regenerants originating from microspore culture were haploid immediately after germination, but only 10 % embryos retained haploidity after 3 years subculturing, while 10.5 % were diploid, 73.5 % tetraploid and 6 % octaploid.

4. DISCUSSION

The smaller horse chestnut microspores (Ćalić et al. 2003) in uninucleate stage are known to be crucial for androgenesis as well as all other plant species (Zheng 2003; Ćalić-Dragosavac et al. 2008, 2009).

In all horse chestnut genotypes examined, the number of embryos formed in the induction media was around five times higher in the suspension of single-nucleus microspores. Our results that the genotype is the most crucial factor for induction androgenic embryo in horse chestnut are correlated with results Höfer (2004) and Höfer et al. (2008).

The maintenance of a low proportion of haploid embryos growth on hormone free medium indicates that significant alternations in the ploidy level had occurred during long-term culture (up to 3 years). The absence of plant growth regulators in the maintenance media of our microspore and anther cultures reduces the possibility of embryo induction from the diploid tissue of the anther wall, favoring the hypothesis of spontaneous diploidisation. Results in other genera, such as *Populus* (Baldursson et al. 1993), *Triticum* (Löschenberger and Heberle-Bors 1992) and *Quercus* (Bueno et al. 2003) corroborate these findings.

Also, the presence of trihoms on cotyledons of androgenic embryos can be explained by the appearance of polyploidy (existence of intensive endoreduplication) in horse chestnut cells, which is in line with results in other plant species (Traas et al. 1998; Joubés and Chevalier 2000).

To the best of our knowledge, this was also the first report on horse chestnut androgenic embryos, presenting influence different genotype on induction androgenesis and flow cytometry data.

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Table 1. *Effect of different genotype (YJ_1-JT_5 and OT_1-OT_5) and age of tree on the induction of androgenic embryos in horse chestnut using anther and microspore culture on MS_1 medium.*

Anther culture						***Microspore culture***	
Genotype	**Age of trees (years)**	**Total N^o of cultivated anthers**	**Embriogenic anthers**		**N^o of embryos/ isolated anthers**		
			N^o	**%**		**N^o anthers**	**embryos/ embryogenic anthers**
YT_1	~ 20	206	35	17.0	3.0 ^{b}	100	16.0 ^{b}
YT_2		210	27	12.9	1.0 ^{c}		4.5 ^{d}
YT_3		203	12	5.9	2.7 ^{b}		9.0 ^{d}
YT_4		209	19	9.1	0.9 ^{c}		5.0 ^{e}
YT_5		201	10	5.0	1.6 ^{bc}		7.0 ^{de}
OT_1	~ 100	208	47	22.6	5.0 ^{a}		27.0 ^{a}
OT_2		210	79	37.6	2.0 ^{bc}		11.0 ^{c}
OT_3		210	68	32.4	1.0 ^{c}		6.0 ^{de}
OT_4		205	75	36.6	1.7 ^{bc}		9.0 ^{d}
OT_5		206	28	13.6	0.5 ^{c}		3.0 ^{e}

*values in each column marked by different letters are significantly different at 0.05 using the SNK test.

Table 2. *Influence of horse chestnut genotypes (YT_1-YT_5 and OT_1-OT_5) and age of tree on appear of albinism in anther and microspore cultures on hormone free medium, on long day (LD) and short day (SD).*

Genotype	Age of tree (years)	Medium	Albino embryos (%)			
			Anther culture		Microspore culture	
			LD	SD	LD	SD
YT_1	~ 20	hormone free	11.0 [b]	23.0 [b]	9.0 [b]	20.0 [b]
YT_2			9.0 [b]	19.0 [c]	7.0 [b]	17.0 [c]
YT_3			7.0 [c]	17.0 [c]	5.0 [c]	15.0 [c]
YT_4			6.0 [c]	15.0 [d]	3.0 [d]	12.0 [d]
YT_5			14.0 [a]	27.0 [a]	11.0 [a]	24.0 [a]
OT_1	~100		4.0 [d]	15.0 [d]	1.0 [e]	11.0 [e]
OT_2			9.0 [b]	22.0 [b]	6.0 [bc]	17.0 [c]
OT_3			7.0 [c]	19.0 [c]	5.0 [c]	15.0 [c]
OT_4			5.0 [d]	17.0 [c]	3.0 [d]	13.0 [d]
OT_5			11.0 [b]	25.0 [a]	8.0 [b]	19.0 [b]

*values in each column marked by different letters are significantly different at 0.05 using the SNK test